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Rapid communication

Molecular characterization of the VLDL receptor homolog mediating binding of lipophorin in oocyte of the mosquito *Aedes aegypti*

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Abstract

Lipophorin (Lp) functions as a yolk protein precursor in the mosquito *Aedes aegypti* and it is internalized via receptor-mediated endocytosis (Insect Biochem. Mol. Biol., 30 (2000) 1161). We cloned and molecularly characterized a putative mosquito ovarian lipophorin receptor (*Aa*LpRov) cDNA. The cDNA has a length of 3468 bp coding for a 1156-residue protein with a predicted molecular mass of 128.9 kDa. The deduced amino acid sequence of the cDNA revealed that it encodes a protein homolog of the LDL receptor superfamily, and that it harbors eight cysteine-rich ligand binding repeats at the N-terminus like vertebrate VLDL receptors. The deduced amino acid sequence of this mosquito ovarian receptor is most similar to that of the locust lipophorin receptor (*Lm*LpR) (64.3%), and is only distantly related to the mosquito vitellogenin receptor (VgR) (18.3%), another ovarian LDLR homolog with a different ligand. The *Aa*LpRov cDNA was expressed in a TnT® Coupled Reticulocyte Lysate system, and co-immunoprecipitation experiments confirmed that the receptor protein specifically binds Lp. Developmental expression profiles clearly showed that *Aa*LpRov transcripts are present in the vitellogenic ovary, with peak expression at 24–36 h post blood meal. In situ hybridization indicated that *Aa*LpRov transcripts are present only in female germ line cells. Distance-based phylogenetic analyses suggest that the insect LpR and vertebrate LDL/VLDL receptor lineages separated after divergence from the insect VgR lineage.
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1. Introduction

In oviparous animals, vitellogenesis is the principal event responsible for oocyte growth. The developing embryo of an oviparous animal draws practically all of its requisite nutrients from a cache of proteins, lipids, and carbohydrates stored within the egg as yolk. In insects, most yolk protein precursors are synthesized in and secreted from the fat body, and are transported as hemolymph proteins to the developing oocyte. Internalization of yolk precursors occurs via receptor-mediated endocytosis.

Receptor-mediated endocytosis, an essential process in all eukaryotes, is required for general cellular functions, including uptake of nutrients (e.g., low-density lipoprotein [LDL] or transferrin) and recycling of membranes and membrane proteins (Mukherjee et al., 1997). Yolk uptake by growing oocytes is a dramatic example of a receptor-mediated endocytosis pathway in many species, including invertebrates such as the nematode *Caenorhabditis elegans* (*C. elegans*) and insects, and vertebrates such as the chicken. Yolk and yolk receptor endocytic trafficking is thought to proceed through pathways very similar to those of LDL and LDL receptors in somatic cells (Schneider, 1996).

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In fish and amphibians, there is no clear evidence that lipoproteins other than vitellogenin (Vg) serve as yolk precursors (Prat et al., 1998). In birds, in addition to Vg, large quantities of very-low-density lipoprotein (VLDL) are incorporated and sequestered in oocytes during vitellogenesis (Prat et al., 1998). Barber et al. (1991) reported that the major plasma proteins, which are targeted to chicken oocytes, are Vg and very low-density lipoprotein (VLDL), a carrier of energy in the form of triacylglycerol. Three ovarian LDL/VLDL receptors have been cloned and characterized from vertebrates — the rainbow trout (Prat et al., 1998), the South African clawed toad (Opresko and Wiley, 1987), and the chicken (Schneider, 1996).

Lipophorin (Lp), the major lipoprotein in insect hemolymph, plays a dual role in lepidopteran vitellogenesis, shuttling precursors from the fat body to the ovaries for the deposition of lipid yolk droplets, and in some species becoming one of the major constituents of the protein yolk bodies (Telfer et al., 1991). The recently characterized locust LpR is a homolog of vertebrate VLDLR's, and is hypothesized to be the endocytic receptor for high-density lipophorin (HDL) (Dantuma et al., 1999). In the yellow fever mosquito, *Aedes aegypti* (*Aa*), lipid accumulation in the oocyte during oogenesis is triggered by the ingestion of a blood meal and the oocyte size increases more than 300 fold within 36 h. Mosquito Lp functions as a yolk protein precursor. Morphological and kinetics studies of Lp accumulation by developing oocytes suggest that it is internalized via receptor-mediated endocytosis (Sun et al., 2000). Sappington et al. (1996) characterized the mosquito vitellogenin receptor (VgR) which, along with the *Drosophila* yolk protein (YP) receptor (Schonbaum et al., 1995), represent a recently discovered subfamily of the LDLR family receptors.

We report herein the cloning and functional analysis of the ovarian lipophorin receptor (*Aa*LpRov) cDNA of the mosquito. This ovarian receptor is clearly a homolog of the locust LpR and vertebrate LDL/VLDL receptors. It is only distantly related to other ovarian Vg/yolk protein receptors that have been cloned from the fruit fly (Schonbaum et al., 1995), the mosquito (Sappington et al., 1996), and the nematode (Grant and Hirsh, 1999). The discovery that developing mosquito oocytes contain *Aa*LpRov in addition to VgR illuminates further the complexity of the receptor endocytic machinery required for successful development of eggs in this insect vector of numerous human pathogens.

2. Materials and methods

Adult yellow fever mosquitoes, *Aedes aegypti* were maintained at 27°C and provided with water and a 10% sucrose solution. Larvae were fed on a standard diet. In order to initiate vitellogenesis, 3–5-day-old females were fed with a blood meal on white rats. The ovaries were dissected from the blood-fed females at 12, 24, 36, 48, and 72 h after the blood meal.

A cDNA fragment of the mosquito LpR was first amplified from a λZAP II cDNA library prepared from previtellogenic female *A. aegypti* mosquitoes using degenerate primers. The degenerate primers were based on conserved regions of vertebrate LDL and VLDL receptors (GeneBank Database). The sense primer was from a conserved region of an EGF homology domain (AVYKANKF; 5′-GC (AT)GT(CG)TATAA(AG)GC (CA)AA(TC)AAATTC-3′). The antisense primer was from the region containing the internalization signal (MNFDNPVY; 5′-GTACAC(ACTG)GGATTGTC(GA) AAGTTCAT-3′). The λZAPII cDNA library was prepared from previtellogenic female mosquitoes as previously reported (Cho et al., 1991). The amplified ~700 bp PCR product was subcloned into the pGEM-T vector and sequenced from both ends, revealing high similarity to LDL and VLDL receptors; thus it was used to screen a λZAPII cDNA library. Several positive cDNA clones were subsequently isolated, and sequencing was performed in the W. M. Keck facility at Yale University. The deduced amino acid sequence was analyzed with DNASTAR software (DNASTAR Inc., Madison, WI).

Total RNA was isolated from mosquito ovaries of different developmental stages using the "RNeasy" mini kit (Qiagen) according to the manufacturer's instructions. For Northern blot analysis, total RNA (10 μg) was subjected to agarose gel electrophoresis in the presence of formaldehyde. The RNA from the gel was capillary blotted onto an Immobilon Ny^+ membrane (Millipore Crop., Bedford, MA), UV cross-linked, and hybridized using a 1.2 kb cDNA fragment. The probe was labeled with ^{32}P by random priming. The hybridization was carried out at 65°C for 16 h with the labeled probe (2×10^6 cpm/ml) using Hybrisol II (Oncor, Gaithersburg, MD) containing 0.5 mg/ml denatured salmon sperm DNA. The blots were washed twice in 1×SSC and 0.1% SDS for 30 min at room temperature, once in 0.1×SSC and 0.1% SDS for 10 min at 65°C, and exposed to Kodak film.

Ovaries were dissected from 3–4-day-old previtellogenic mosquitoes and processed for hybridization by the method of Suter and Steward (1991). A single-strand antisense cDNA probe was prepared by incorporating digoxigenin-11-dUTP into a 5.5 kb *Aa*LpRov cDNA fragment with PCR as described by N. Patel and C. Goodman in the Roche Biochem Applications Manual, and in the Roche Biochem Genius System User's Guide. Sense strands were prepared as controls in the same way. To facilitate penetration into the ovary, the size of the DNA probe was reduced by boiling for 1 h in hybridization solution. After hybridization, ovaries were incubated with alkaline phosphatase-conjugated anti-digoxigenin antibodies, and the bound complexes visualized by incubation with NBT/BCIP solution (all from Roche Biochem) for 10–20 min at room temperature. Ovarioles were isolated and mounted in Gel/Mount (Biomeda, Foster City, CA).

To characterize *Aa*LpRov functionally, we expressed the receptor protein. The full-length cDNA was subcloned into a pBluescript II vector (Stratagene), and the plasmid DNA was prepared with the QIAfilter plasmid kit (Qiagen). The expression of *Aa*LpRov was conducted in the TnT® Coupled Reticulocyte lysate system according to the manufacturer's instruction (Promega). Protein labeling was performed by incorporation of ^{35}S-methionine during the translation. Binding of the expressed protein was analyzed by co-immunoprecipitation. Labeled or unlabeled Lp was prepared by the method previously reported (Sun et al., 2000). Binding reactions between the 2.0 μl expressed product and the 70 μl of fat body culture medium was carried out in an incubation buffer for 1 h at 4°C. Ten microliters of polyclonal antibodies against the Lp large subunit were added to the reaction mixture and incubated for 3 h at 4°C. Then 30 μl protein A-Sepharose (Boehringer Mannhein) were added and incubated for another 4 h at 4°C. The binding complexes were precipitated by centrifugation and washed three times in TBS-T buffer (20 mM Tris–HCl, pH 7.5 containing 0.15 M NaCl, 0.05% Tween 20). Finally, bound protein was visualized by 10% SDS–PAGE and fluorography.

3. Results and discussion

To identify an *Aa*LpRov cDNA, two degenerate primers were designed and synthesized as described in Materials and Methods. Using these degenerate primers, a ~700 bp PCR fragment was obtained from the cDNA library, subcloned into a pGEM-T vector (Promega), and further analyzed. The deduced amino acid sequence of the PCR fragment indicated that it was highly similar to vertebrate LDL and VLDL receptors. It was then used to clone the LpR cDNA from the cDNA library. The screening resulted in the identification of a full-length cDNA insert, encoding the entire *Aa*LpRov sequence.

Sequencing of *Aa*LpRov defined an open reading frame of 3468 bp coding for a 1156-residue protein (Accession No.: AF355595). This open reading frame predicts a molecular mass of 128.9 kDa and an isoelectric point of 5.1. The putative methionine start codon (ATG) is preceded by several in-frame stop codons, and the sequence downstream codes for a typical signal peptide with a length of 23 amino acids, indicating that this sequence represents the full-length open reading frame. A stretch of 23 predominantly hydrophobic residues defines a cleavage site for the signal peptide. Another hydrophobic region, presumably representing a transmembrane domain, is found at amino acid residues 1077–1098.

According to the deduced amino acid sequence, the protein encoded by the full-length cDNA is a member of the LDLR family (Figs. 1 and 2). There are five recognizable domains: I) the first encodes the putative ligand-binding domain consisting of eight cysteine-rich Class A repeats (LR8) like the vertebrate VLDLR. II) The EGF-precursor domain contains three EGF-precursor repeats and five copies of the characteristic YWXD sequence, spaced by approximately 50 amino acid intervals. III) The third domain is a stretch of 257 amino acid residues between the EGF-precursor domain and the transmembrane domain, which is serine and threonine rich, thus containing multiple potential sites for O-linked sugar chains. IV) The fourth domain is a single membrane-spanning domain consisting of a hydrophobic stretch of 22 amino acids. V) The putative cytoplasmic tail of the receptor contains a highly conserved internalization signal (amino acid 1111–1116) FDNPVY required for direction the human LDL receptor to clathrin-coated pits. Interestingly, this receptor contains the crucial tyrosine residue in the internalization signal, which was absent in the insect Vg/YP receptors (Schonbaum et al., 1995; Sappington et al., 1996).

The *Aa*LpRov has a high structural similarity to the locust lipophorin receptor (*Lm*LpR) (Fig. 2) (Dantuma et al., 1999). The modular arrangement of the three classes of repeats (Class A cysteine-rich repeat, Class B cysteine-rich repeat, and YWXD repeat) in both insect receptors is typical of those found in other LDLR family molecules (Figs. 2 and 3). The ligand binding domain, the EGF precursor domain, and the cytoplasmic domain are highly conserved between these two species. A sequence of a putative LpR receptor with a similar protein modular domain structure is present in the genome of *Drosophila* (Acession No.: AE003753_10 CG4823). The ligand-binding domain in *Aa*LpRov consists of eight Class A ligand binding repeats, thus making it similar to vertebrate VgR and VLDLR (Fig. 2A). The chicken VgR is an 8-repeat VLDL-type receptor expressed in the ovary (Schneider, 1996). It also has been reported that the human ovary expresses an 8-repeat receptor (Hussain et al., 1999). The Vg/YPRs of the mosquito (Sappington et al., 1996) and *Drosophila* (Schonbaum et al., 1995) are 13-repeat receptors, with class A repeats divided into an N-terminal cluster of five and a more membrane-proximal cluster of eight (Fig. 2A). Interestingly, the newly discovered RME-2 gene which encodes the nematode VgR in the oocyte of *C. elegans* is the novel 5-repeat member of the LDLR gene family (Grant and Hirsh, 1999) (Fig. 2A).

The *Aa*LpRov is quite different from the *Lm*LpR in the length of its putative O-linked sugar domain. *Aa*LpRov has an unusually long (257-residues) putative O-linked sugar domain. *Lm*LpR′ domain is only 30-residue long, a length similar to that of the *Aa*VgR (Fig. 2A). Many LDLR-family proteins contain an O-linked sugar

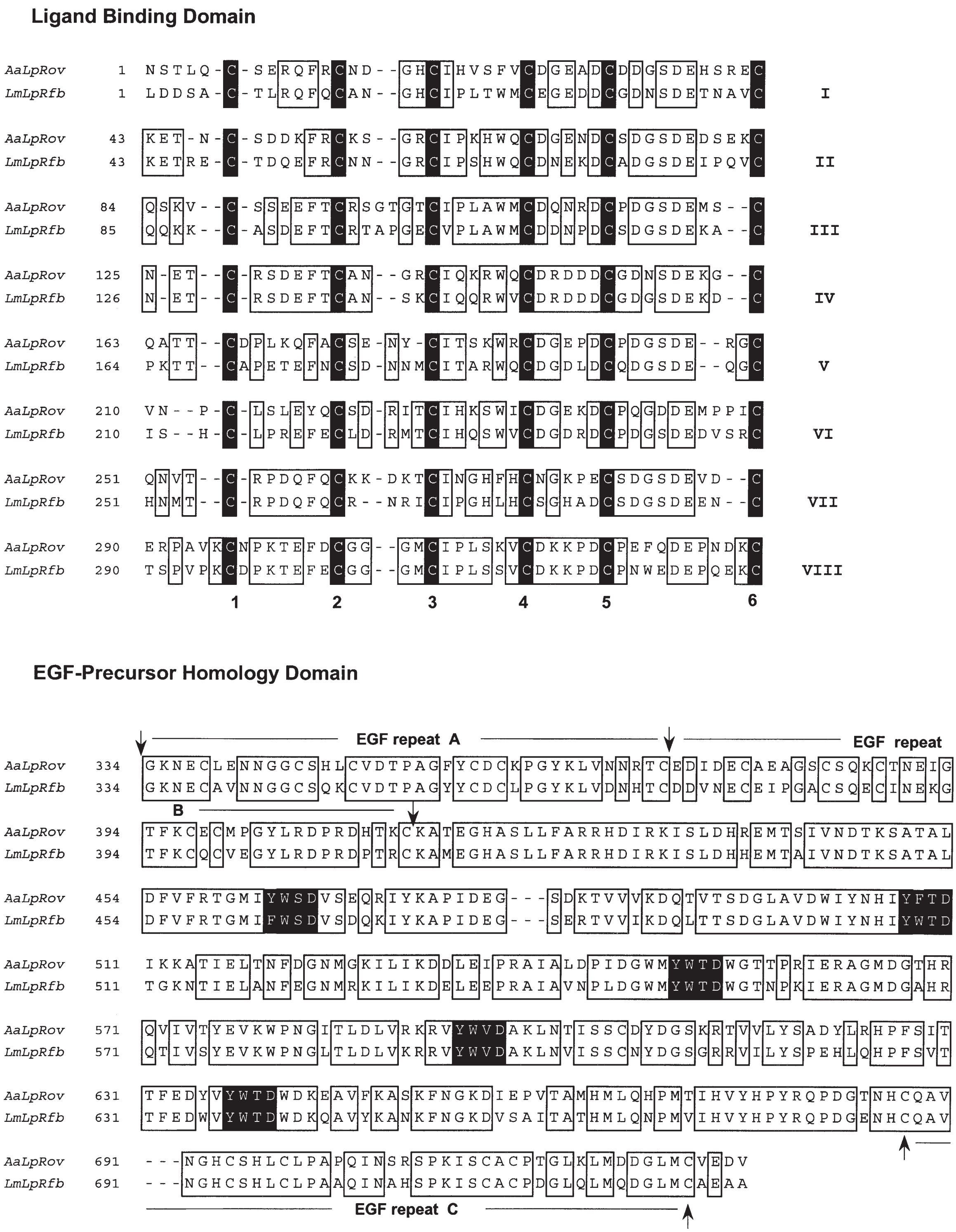

Fig. 1. The amino acids in the five functional domains of mosquito lipophorin receptor (*Aa*LpRov) were compared with those in the locust lipophorin receptor (*Lm*LpR). The eight cysteine-rich repeats (I–VIII), that compose the ligand-binding domain, are aligned. The cysteine residues (1–6) are indicated by reverse phase. The EGF-precursor A, B, and C repeats are aligned. The F/YWXD sequences (reverse phase), located between EGF-precursor repeat B and C are indicated. In the putative O-linked sugar domain, threonine and serine residues are presented in reverse phase. The cytoplasmic tail contains the conserved internalization signal FDNPVY (dark shading). Amino acids are numbered on the left and identical amino acids are boxed.

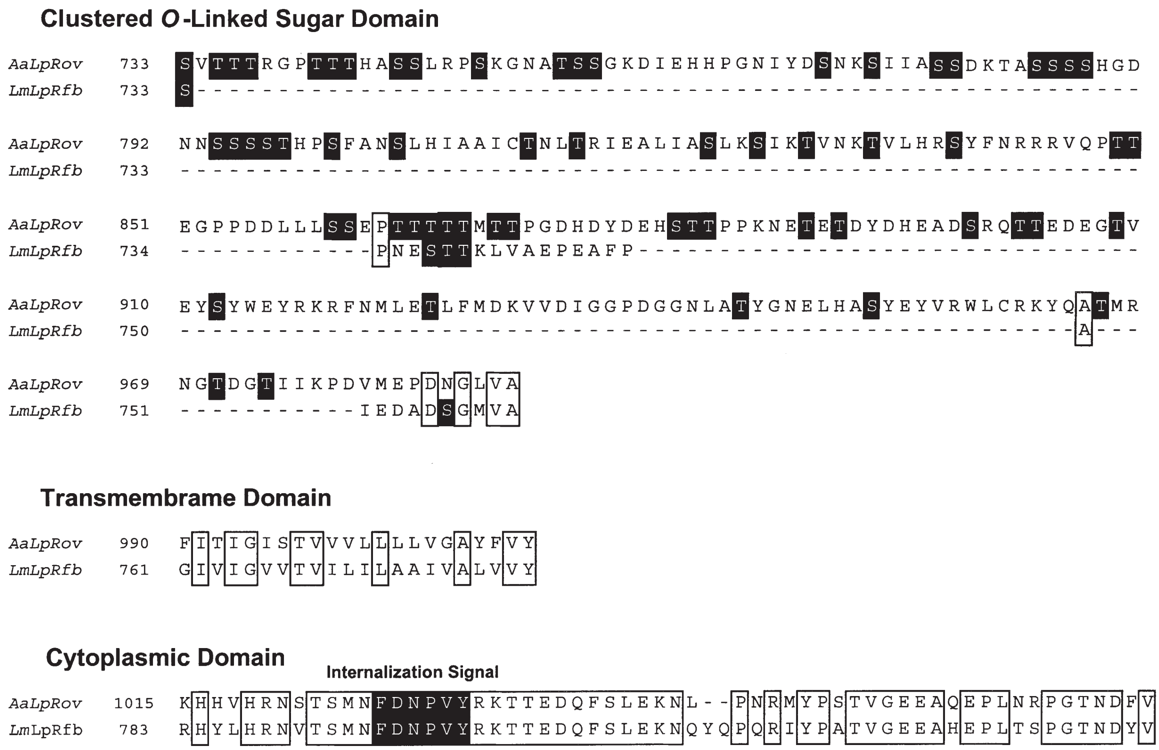

Fig. 1. (*continued*)

domain in this same relative position, but others do not. The O-linked sugars may serve to protect the receptor from membrane-bound proteases. Alternative splicing of the chicken VgR/LR8 gene produces an oocyte-specific VgR without an O-linked sugar domain, or a soma-specific variant containing a 30-residue O-linked region (reviewed in Sappington and Raikhel, 1998a).

The mosquito LpR differs structurally from the mosquito VgR in having only a single cluster of class A ligand binding repeats rather than two, three class B EGF-type repeats rather than seven, and only a single YWXD domain rather than three. In addition, the amino acid identity between the two receptors is fairly low (18.3%). This is reflected in the distance-based phylogenetic reconstruction (Fig. 2B), in which the insect LpR's are seen to be more only distantly related to insect VgR/YPR's. In contrast, the insect LpR's share a much more recent common ancestor with vertebrate LDLR's and VLDLR/VgR's. Based on amino acid fingerprint analyses, Sappington and Raikhel (1998b) previously proposed that seven-repeat LDLR's arose from an eight-repeat ancestor through the loss of the latter's first module. This conclusion is supported by the reconstruction of LpR phylogeny (Fig. 2B) which suggests that the common ancestor of LpR's, LDLR's, and VLDLR's contained an eight-repeat class A cluster, and that the seven-repeat LDLR's represent the derived form. The nematode VgR (RME-2) amino acid sequence is quite divergent from all other sequences tested. Its five-repeat cluster is reminiscent of the five-repeat cluster in the insect VgR/YPR's, but assignment of homology based solely on the number of repeats could be misleading (Sappington and Raikhel, 1998b). A more detailed phylogenetic study awaits amino acid sequence fingerprint analyses of the nematode VgR and the insect LpR's.

Temporal expression of *Aa*LpRov mRNA was examined in mosquito ovaries during previtellogenic and vitellogenic periods of egg development. Northern blot analysis, using a 1.2 kb fragment from an *Aa*LpR cDNA clone as a probe, demonstrated that there are 4.5 kb transcripts in ovarian preparations (Fig. 3A). During all vitellogenic stages, *Aa*LpRov transcript was clearly detectable. In the previtellogenic stage, *Aa*LpRov transcript was present and increased further after the onset of vitellogenesis, peaking by 24 h post blood meal (PBM), when yolk protein gene transcription is nearing its maximum (Sappington and Raikhel, 1998a) and the endocytic activity of oocytes is at its highest point. The *Aa*LpRov mRNA was present in the ovary until 48 h PBM, the time of termination of vitellogenic events in the female mosquito. The pattern of *Aa*VgR mRNA expression in the ovaries is similar to that of *Aa*LpRov, but *Aa*VgR mRNA was expressed at higher levels in both previtellogenic and early vitellogenic ovaries. The developmental expression of both transcripts in the ovary supports their putative functions in coding for receptors that mediate Lp or Vg uptake by the ovary.

The spatial distribution of the *Aa*LpR transcript in the ovary was demonstrated by whole mount in situ

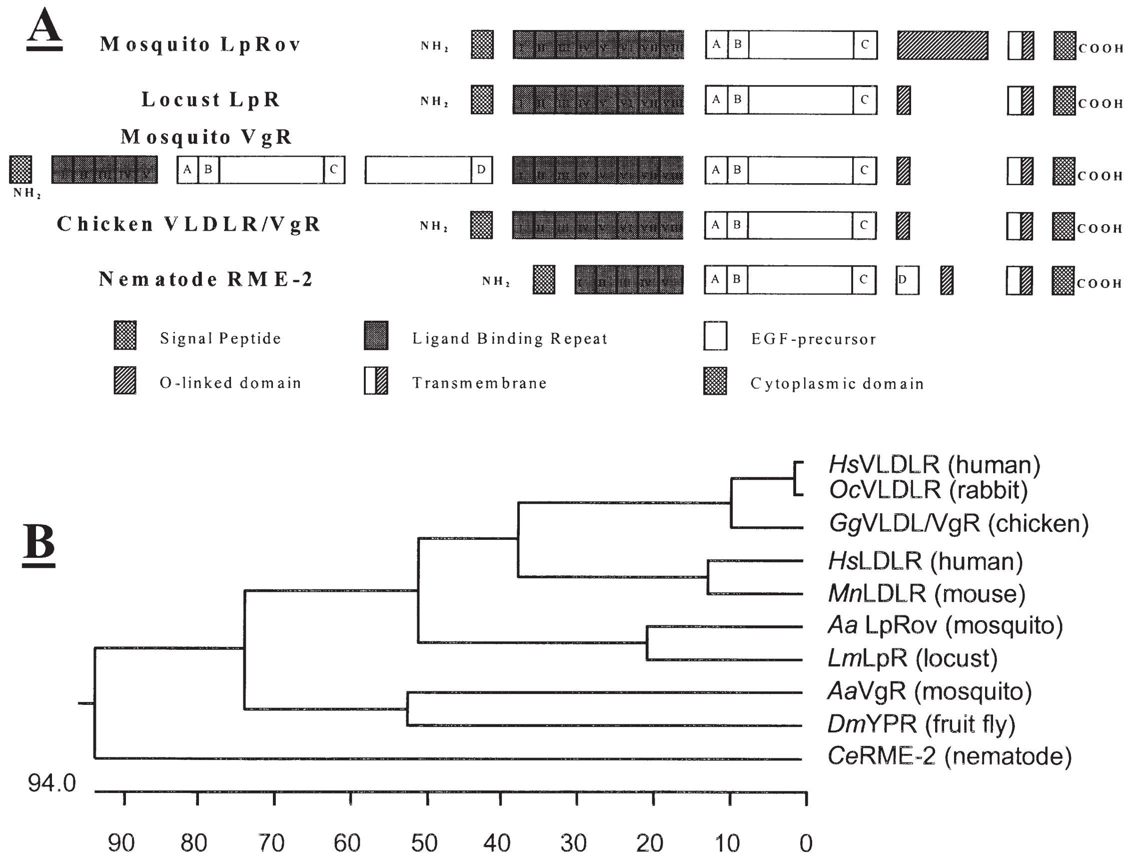

Fig. 2. Schematic modular domain alignment and phylogenetic relationships of *Aa*LpRov with other LDLR superfamily members. (A) Schematic alignment of *Aa*LpRov modular domains with locust LpR, mosquito VgR, and nematode RME-2. The cysteine-rich repeats in the ligand binding domains and EGF precursor domains are assigned the Roman numerals I–VIII and the letters A–D, respectively. Locust LpR, *Locust migratoria* lipophorin receptor (Dantuma et al., 1999); Mosquito VgR, *Aedes aegypti* VgR (Sappington et al., 1996); Chicken VLDLR/VgR, *Gallus gallus* VLDLR/VgR (Schneider, 1996); Nematode RME-2, *Caenorhabditis elegans* VgR (Grant and Hirsh, 1999). (B) Phylogenetic relationships among 10 LDLR superfamily members. Whole sequences from multiple species were aligned with the PAUP program (Swofford, 1993). Lengths of branches along the horizontal axis are proportional to evolutionary distances calculated from the amino acid identity matrix. *Aa*VgR from *Aedes aegypti* (accession no. L77800); *Lm*LpR from *Locust migratoria* (accession no. AJ000010), *Gg*VLDL/VgR from *Gallus gallus* (accession no. 159570), *Hs*VLDLR from *Homo sapiens* (accession no. S73849), *Hs*LDLR from *Homo sapiens* (accession no. NM000527), *Mn*LDLR from *Mus musculus* (accession no. P35951), *Oc*VLDLR from *Oryctolagus cuniculus* (accession no. 547843), *Dm*YPR from *Drosophila melanogaster* (U13637), *Ce*RME-2 from *Caenorhabditis elegans* (AF185706).

hybridization. A control (sense) DNA probe did not produce any hybridization signal in the mosquito ovary at any stage of development (Fig. 3B), confirming the specificity of the hybridization technique used in this study. The hybridization signal produced by the antisense specific probe was observed in substantial amounts in oocytes of developing primary follicles, whereas nurse cells exhibited considerably lower levels of the *Aa*LpR transcript (Fig. 3B2 and 3B3). The *Aa*LpRov transcript was not present in the somatic follicle cells.

The *Aa*LpR transcript was detected in the ovary from the first day of previtellogenic development of the females and throughout the vitellogenic stage; the distribution of the signal was similar at every stage of vitellogenesis, being present in the oocyte and nurse cells of primary follicles. The overall distribution of the *Aa*LpR transcript in the mosquito ovary was the same as that previously described for *Aa*VgR (Sappington et al., 1996) and *Aa* clathrin heavy chain transcripts (Kokoza and Raikhel, 1997). In the *Drosophila* ovary, the YP receptor transcript also was localized in the oocyte and nurse cells of each follicle (Schonbaum et al., 2000). Transgenic study of the gene encoding the *Drosophila* YP receptor has demonstrated that it is expressed only in the nurse cells of developed follicles and its transcript is accumulated in developing oocytes (Schonbaum et al., 2000). In contrast, protein products of genes encoding *Aa*VgR, *Aa* clathrin heavy chain, and *Drosophila* YP receptor were found exclusively in oocytes (Sappington et al., 1996; Kokoza and Raikhel, 1997; Schonbaum et al., 2000). This suggests that there is an additional regulatory step at the translational level allowing these mRNAs to be translated only after they are transported into developing oocytes.

Interestingly, *Aa*LpRov mRNA was also detected in the germarium where it is localized in germ-line cells as

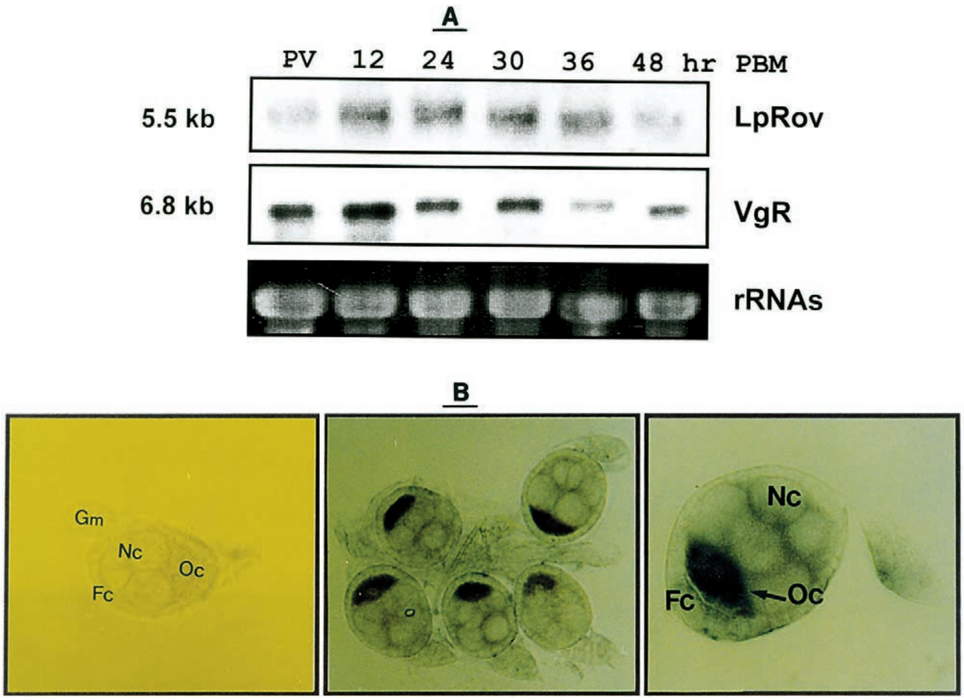

Fig. 3. Developmental profile and tissue-specific expression of *Aa*LpRov transcripts. (A) *Aa*LpRov and *Aa*VgR mRNA transcripts in the ovary throughout oogenesis. For Northern blot analysis, total RNA was extracted from the ovaries of female mosquitoes at the indicated times. A blot was probed with a ^{32}P-labeled *Aa*LpRov and *Aa*VgR cDNA fragments. Ribosomal RNAs (rRNAs) are shown as an internal control after staining with ethidium bromide. PV, previtellogenic stage; PBM, post-blood meal. (B) In situ hybridization of *Aa*LpRov mRNA in 3–4-day-old previtellogenic follicles. Fixed ovaries were incubated with extensively boiled digoxygenin-labeled cDNA probe followed by incubation with alkaline phosphatase-conjugated anti-digoxigenin antibody. *Aa*LpRov mRNA was visualized colorimetrically by incubating with NBT/BCIP. B1, Control follicle probed with sense cDNA. B2 and B3, Follicle probed with antisense cDNA. Oc, oocyte; Nc, nurse cells; Fc, follicle cells; Gm, germarium.

well as in secondary follicles separated from the germarium at mid-vitellogenesis (data not shown). A similar phenomenon has been observed for the *Aa*VgR and *Aa* clathrin heavy chain transcripts, (Sappington et al., 1996; Kokoza and Raikhel, 1997). Likewise, in the *Drosophila* ovary, the YP receptor transcript was detected in germline cells and early previtellogenic follicles in which it was present in the cells of the germ-line lineage, oocytes and nurse cells (Schonbaum et al., 2000). Thus, it appears that the expression of genes coding components of the oocyte receptor-mediated machinery is specifically activated in the germ-line cells as a part of the developmental program of ovarian differentiation.

Experiments utilizing the protein product of the in vitro-expressed *Aa*LpRov cDNA strongly suggested that it indeed encoded a specific receptor for Lp. When the [^{35}S]-methionine-labeled in vitro-expressed *Aa*LpRov was incubated with anti-Lp antibodies alone, the antibodies did not precipitate the receptor (Fig. 4, lane 1). In a specificity control, in vitro metabolically [^{35}S]-methionine-labeled Lp was immunoprecipitated by anti-Lp antibodies (Fig. 4, lane 2). The [^{35}S]-methionine-labeled in vitro-expressed *Aa*LpRov was first incubated with unlabeled Lp and then immunoprecipitated with anti-Lp antibodies; SDS–PAGE followed by autoradiography revealed *Aa*LpRov as the immunoprecipitated product (Fig. 4, lane 3). An estimated size of 140 kDa for the immunoprecipitated protein was the same as that of [^{35}S]-methionine-labeled in vitro-expressed *Aa*LpRov which was not subjected to any treatment (Fig. 4, lane 4). Thus, co-immunoprecipitation utilizing anti-Lp antibodies demonstrated that the cloned *Aa*LpRov specifically bound Lp purified from the secretions of vitellogenic fat bodies. In our preliminary binding experiments utilizing solubilized ovarian membrane proteins from female mosquitoes, we have shown that the ovary contains a putative receptor protein that binds Lp in a saturable manner and is distinct from VgR (Sun, J. and Raikhel, A.S., unpublished data).

Thus, it appears that the receptor-mediated machinery, which is responsible for a highly specific and rapid accumulation of yolk protein precursors by mosquito oocytes, contains at least two separate receptors, VgR and LpR, which are specific for their respective ligands, Vg and Lp. Mosquito oocytes, however, have been shown to accumulate two other yolk protein precursors, the pro-enzymes vitellogenic carboxypeptidase (VCP) and vitellogenic cathepsin B (VCB) (Cho et al. 1991, 1999). Future studies should demonstrate whether there are specific receptors for VCP and VCB present in mosquito oocytes, or alternatively if these ligands are internalized via already known receptors, VgR or LpR.

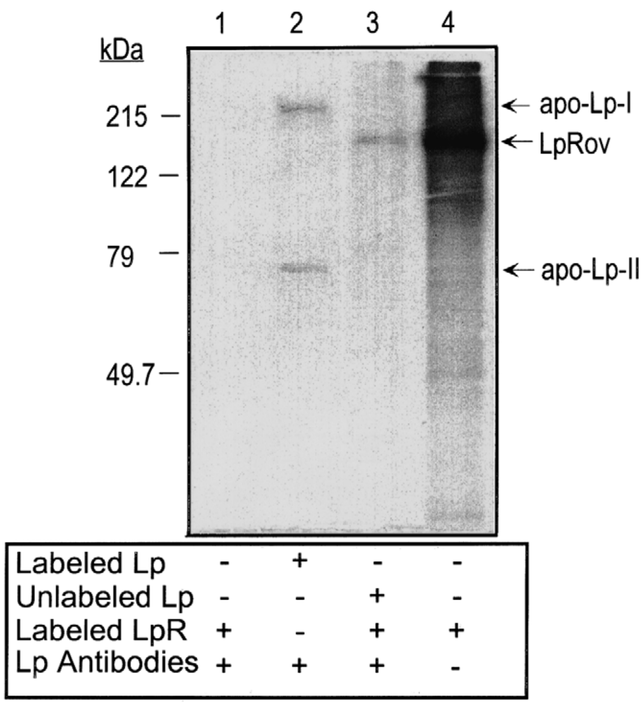

Fig. 4. *Aa*LpRov cDNA expression and functional co-immunoprecipitation analysis. The *Aa*LpRov cDNA was expressed using the TnT® Coupled Reticulocyte lysate system. Lane 1 — [^{35}S]methionine-labeled expressed *Aa*LpRov immunoprecipitated with anti-Lp antibodies; lane 2 — in vitro metabolically [^{35}S]methionine-labeled Lp immunoprecipitated with anti-Lp antibodies; lane 3 — [^{35}S]methionine-labeled expressed *Aa*LpRov incubated with unlabeled Lp and then immunoprecipitated with anti-Lp antibodies; lane 4 — [^{35}S] methionine-labeled expressed *Aa*LpRov used in lanes 1 and 3 for immunoprecipitation experiments. Arrows indicate positions of apo-Lp-I, apo-Lp-II and LpR. Numbers on left are molecular mass markers (Bio–Rad).

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